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Cold Shock Proteins Contribute to the Regulation of Listeriolysin O Production in *Listeria monocytogenes*

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Abstract

Cold shock proteins (Csps) are multifunctional nucleic acid binding proteins used to regulate a wide range of gene expression responses in bacteria. We report here that Csps regulate the production of the pore-forming cytolysin listeriolysin (LLO) and hemolysis phenotypes in *Listeria monocytogenes*. A triple *csp* gene deletion mutant incapable of producing any Csps, as well as double *csp* gene deletion mutants only producing either CspA or CspD, caused less hemolysis and produced lower LLO concentration. On the other hand, another double *csp* gene deletion mutant that produces CspB retained hemolysis and LLO production levels that are similar to the parental wild-type strain. Transcription analysis showed that in absence of all three *csp* genes or *cspB* alone, *L. monocytogenes* cells have decreased levels of *hly* gene transcripts, which code for the synthesis of LLO proteins. A comparative examination of mRNA stability showed that *hly* transcripts were more rapidly degraded in *L. monocytogenes* triple *csp* gene deletion mutant cells that are not capable of producing Csps. Overall, our results indicate that Csps, in particular CspB, are important components of gene expression regulatory mechanisms that promote efficient LLO production and hence virulence responses of *L. monocytogenes*.

Introduction

Listeria monocytogenes is a widespread Gram-positive foodborne pathogen associated with rare but serious public health problems. Infections of neonates and immunocompromised adults cause severe clinical illnesses and high mortality, whereas in pregnant women such infection leads to abortions (Posfay-Barbe and Wald, 2009; Allerberger and Wagner, 2010). In addition, this bacterium is an important concern to the food industry where it is associated with food safety control challenges and substantial economic losses (Kramer *et al*., 2005; Gandhi and Chikindas, 2007). In particular, regular food hygiene measures that are used to eliminate pathogens from the human food supply are hampered by both the ubiquity and robust stress-resistance phenotypic properties of *L. monocytogenes* (Gandhi and Chikindas, 2007). These properties do not only allow this pathogen to survive regular food hygiene measures, but also to multiply on food products, including those that are conserved by refrigeration and inclusion of food preservatives.

The virulence and stress resistance displayed by *L. monocytogenes* are important attributes enabled through various molecular response mechanisms that are at its disposal (Chaturongakul *et al*., 2008; Dussurget, 2008; Freitag *et al*., 2009; Soni *et al*., 2011). Important virulence factors include internalins (*inlA* and *inlB*), listeriolysin O (LLO), and actin polymerization-inducing proteins (ActA). The internalins are surface-associated proteins used by this pathogen to facilitate invasion of non-phagocytic target host cells (Gaillard *et al*., 1991; Dramsi *et al*., 1995). LLO is a pore-forming cytolysin, which is crucial for intracellular survival because it allows *L. monocytogenes* to escape from internalization vacuoles into the host cell cytosol where replication occurs (Cossart *et al*., 1989; Kayal and Charbit, 2006). The actin polymerization inducing protein ActA is essential for cytosol movement and cell-to-cell spread (Kocks *et al*., 1992).

One of the important stress-protection mechanisms in this bacterium involves production of proteins of the cold shock protein A (CspA) family that was first described in *Escherichia coli*. These are highly conserved, small multifunctional nucleic acid binding proteins that regulate a wide range of gene expression responses in microorganisms (Graumann and Marahiel, 1998; Phadtare, 2004; Phadtare and Inouye, 2004; Horn *et al*., 2007). Cold shock proteins (Csps) mediate their functions through modulation of transcription and translation processes and mRNA stability (Feng *et al*., 2001; Phadtare and Inouye, 2001; Phadtare and Severinov, 2010).

L. monocytogenes possesses three Csps (CspA, CspB, and CspD) that have been shown to promote both cold and osmotic stress adaptation responses (Schmid *et al*., 2009). In addition, there is also experimental evidence suggesting that Csps are involved in modulation of virulence-associated responses in *L. monocytogenes*. In a previous transcriptome analysis study, it was found that *cspA* and *cspB* genes were

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among other genes activated during host infection (Camejo *et al*., 2009). Moreover, Csps have been found to facilitate oxidative stress resistance and host cell invasion (Loepfe *et al*., 2010). *L. monocytogenes* mutant strains deleted in *csp* genes (Δ*cspBD* and Δ*cspABD*) were diminished in host cell infectivity and showed increased oxidative stress susceptibility. At present the Csp controlled cellular mechanisms that might facilitate *L. monocytogenes* virulence are not yet understood. However, based on these previous observations, one hypothesis was that Csps might promote intracellular oxidative stress survival of *L. monocytogenes* during host cell infection (Loepfe *et al*., 2010). In addition to promoting anti-oxidative responses, it is also possible that Csps might influence virulence processes through regulating the expression of specific virulence proteins in this bacterium. Thus, some of the proteins that are critical in *L. monocytogenes* virulence such as LLO might be subjected to expression regulation through Csp-dependent regulatory pathways. In the present study, we show that the expression of the key virulence protein LLO is also subjected to Csp-dependent regulation.

Materials and Methods

Bacterial strains and growth conditions

The *L. monocytogenes* EGDe strains used in this study are listed in Table 1. The *csp* gene deletion mutant variants of *L. monocytogenes* EGDe were constructed as previously described (Schmid *et al*., 2009). The strains were grown overnight in 10 mL of Brain Heart Infusion broth (BHI; Oxoid, Hampshire, UK) at 37°C with shaking at 225 rpm. These primary cultures were diluted (1:100) in fresh BHI media and similarly regrown to give secondary cultures used for experiments. To compare *hly* and *prfA* transcripts, the cultures were diluted to an optical density $(OD)_{590}$ of 0.01 and grown to the stationary (OD_{590} 1.4) growth stage. To compare *hly* mRNA stability, wild-type and Δ*cspABD* strains were diluted and grown at 37°C as described above. Transcription was stopped by addition of rifampicin (50 μg/mL; Sigma-Aldrich, Buchs, Switzerland) to midexponential (OD_{590} 0.5) growth stage cultures, and samples were collected at 0, 5, 10, 20, and 30 min.

RNA isolation, reverse transcription, and quantitative real-time polymerase chain reaction (RT-PCR)

Sample aliquots of 2 mL were collected from stationary growth phase or rifampicin-treated mid-log-phase cultures and stabilized using RNAprotect® Bacteria reagent (Qiagen, Hombrechtikon, Switzerland). RNA isolation and cDNA synthesis were performed as previously described (Arguedas-Villa *et al*., 2010). Quantitative RT-PCR was carried out in the Light Cycler 480 instrument (Roche Molecular Diagnostics, Rotkreuz, Switzerland) using the primers listed in Table 2. Reactions were performed in 10 μL, which contained 4 ng cDNA (1:50 dilution) template, 0.5 μM of primers, and LightCycler® 480 SYBR Green I Master mix. The *16S* RNA gene was used as a normalizer for the relative quantification of *hly* and *prfA* mRNAs using the Light Cycler 480 Relative Quantification Software (Roche Molecular Diagnostics, Rotkreuz, Switzerland).

Table 1. Bacterial Strains

Strain	*Genotype*	*Genetic deletion*	*Reference*
EGDe	Wild type	None	
ΔcspAB	*ΔcspAB*	*cspA* and *cspB*	Schmid *et al*., 2009
ΔcspAD	*ΔcspAD*	*cspA* and *cspD*	Schmid *et al*., 2009
ΔcspBD	*ΔcspBD*	*cspB* and *cspD*	Schmid *et al*., 2009
ΔcspABD	*ΔcspABD*	*cspA*, *cspB* and *cspD*	Schmid *et al*., 2009

Hemolytic assays

The *Listeria* cultures prepared as described above were adjusted to solutions containing equal *Listeria* cell concentrations using BHI broth and based on their OD_{590} measurements. To assess hemolysis caused by each strain on Columbia blood agar, 5 μL of these standardized cultures were spotted on Columbia blood agar plates and incubated 24 h at 37°C. To measure secreted hemolytic activities, 2 mL of standardized cultures were centrifuged (2 min at 10,000 × *g*). Supernatants were sterile filtered and reduced by adding 5 mM dithiothreitol (DTT) and 1 h incubation at 37°C. Reduced supernatants (100 μL) were mixed with equal amounts of 0.05% defibrinated bovine blood (Bioswisstec AG, Schaffhausen, Switzerland) diluted in phosphate-buffered saline (PBS) (pH 7.4) and incubated 40 min at 37°C in 96-well microtiter plates. At the end of this incubation, the absorbance of the samples was measured in the SpectraMax® Plus^{384} Microplate Reader (Molecular Devices GmbH, Biberach an der Riss, Germany) set at 540 nm. Bovine blood mixed with PBS instead of EGDe culture supernatants served as negative controls, and their absorbance was set to 100%.

LLO Western blot analysis

Overnight *L. monocytogenes* cultures were standardized based on OD_{590} measurements to equal concentrations of *Listeria* cells using BHI broth and centrifuged (2 min at 10,000 × g). Proteins in the supernatant fractions were precipitated using 10% trichloroacetic acid, washed three times using cold acetone, and resuspended in Laemmli Sample Buffer (Biorad, Cressier, Switzerland). Resuspended proteins were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blot analysis using a primary rabbit polyclonal anti-LLO antibody (Diatheva, Fano PU, Italy) and an alkaline phosphatase conjugated anti-rabbit IgG (Sigma-Aldrich, Buchs, Switzerland) secondary antibody.

Statistical analysis

Statistical analyses were performed using the JMP statistical software (version 8.0; SAS Institute, Cary, NC). All experiments were performed independently at least three times, and within each experiment samples were analyzed in triplicates. The Kruskal-Wallis/Wilcoxon's rank-sum tests were used to determine the statistical significance of differences detected between *csp* mutants and wild-type EGDe strain. *P*-values < 0.05 were considered significant.

Results

Diminished hemolysis and LLO gene expression in EGDe ΔcspABD

We initially observed that an *L. monocytogenes* EGDe deletion mutant lacking *csp* genes causes diminished hemolysis

Table 2. Oligonucleotide Primers[a] Used

Gene	*Name*	*Sequence*	*Reference*
16S rDNA	16S rRNA-fw 16S rRNA-rv	CTTCCGCAATGGACGAAAGT CTCATCGTTTACGGCGTG	Michel *et al*., 2011
hly	hly-fw hly-rv	ACCTCGGAGACTTACG TCCTCCAGAGTGATCG	This study
prfA	prfA-fw prfA-rv	TGGTATCACAAAGCTCACG GACCGCAAATAGAGCC	This study

[a]Oligonucleotide primers were designed using the LC Probe design software (Roche Molecular Diagnostics, Penzberg, Germany) and were synthesized by Microsynth (Balgach, Switzerland).

compared to the parental wild-type strain on blood agar plates (Fig. 1A). These observations were subsequently confirmed by also testing hemolysis caused by secreted LLO in culture supernatants. Compared to the wild type, supernatants recovered from EGDe Δ*cspABD* exhibited fourfold less hemolysis on average when added to PBS suspended bovine red blood cells (Fig. 1B). Reduced hemolytic activity displayed by the Δ*cspABD* strain thus suggested that LLO gene expression was compromised in the absence of Csp functions. The levels of LLO encoding transcripts derived from the *hly* gene were also compared between the wild-type and Δ*cspABD* strains. Results presented in Fig. 1C showed that *hly* encoded mRNA levels in EGDe Δ*cspABD* were also lower ($p < 0.05$; Wilcoxon/Kruskal–Wallis rank-sum test) than those in the wild-type strain. LLO is expressed under positive control of the virulence gene cluster master regulatory protein PrfA in *L. monocytogenes*. To rule out the possibility that reduced *hly* mRNA levels in Δ*cspABD* was caused by impaired PrfA expression, the *prfA* expression between this mutant and the wild-type was also compared. Similar *prfA* mRNA levels were detected in both strains, indicating that low *hly* mRNA levels detected in the Δ*cspABD* were not due to altered *prfA* expression (Fig. 1D).

Role of individual Csps in LLO expression regulation

The potential functional contribution of each of the three *csp* genes to the regulation of LLO production in *L. monocytogenes* EGDe was initially examined using single *csp* gene

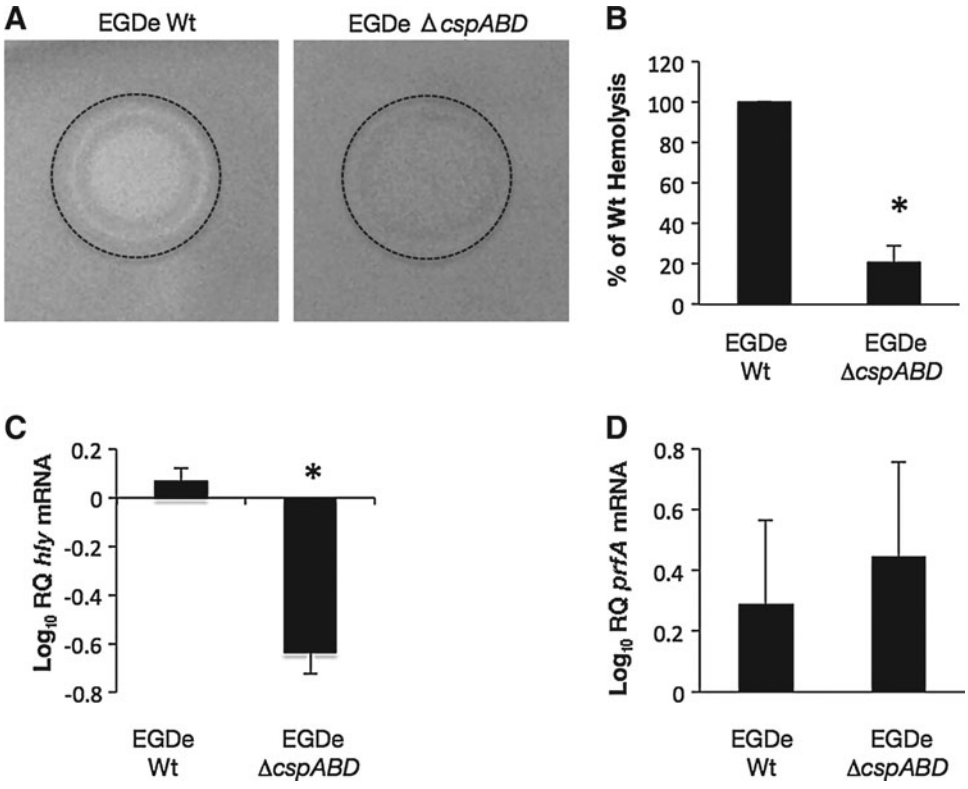

FIG. 1. Hemolysis and gene expression analysis in EGDe wild-type (Wt) and Δ*cspABD* strains. **(A)** Hemolysis observed on Columbia blood (5%) agar. **(B)** Relative quantification of secreted listeriolysin O hemolytic activity. The hemolysis was determined by adding cell-free supernatants to a 0.025% blood solution prepared in phosphate-buffered saline. The hemolytic activities are expressed relative to that of the EGDe wild-type strain (100%). Relative quantification of **(C)** *hly* mRNA and **(D)** *prfA* mRNA levels. Results presented are the means of three independent experimental runs. Error bars indicate the standard error of the mean. An asterisk above the bar denotes statistically significant ($p < 0.05$; Wilcoxon/Kruskal–Wallis rank-sum test) differences in *hly* mRNA levels between the EGDe wild-type and Δ*cspABD* strains.

deletion mutant variants of this strain. None of the single gene deletion mutants of each of the three *csp* genes caused observable phenotypic defects in hemolysis on blood agar plates (data not shown). Based on these results, we concluded that the impact of individual *csp* genes in LLO production in single *csp* gene deletion mutant backgrounds was masked due to potential functional redundancies among the three Csps found in this bacterium. Considering this caveat, we thus opted to use EGDe double *csp* gene deletion mutant variants, which allowed analysis of the role of individual Csps on LLO regulation in backgrounds that are free from functional interference from the other Csps. The hemolysis induced by double *csp* deletion mutants and the wild-type EGDe strain on blood agar plates is shown in Figure 2A. Double-deletion mutant strains harboring either *cspA* or *cspD* similarly also caused significantly less hemolysis than the parental wild-type strain. A double-deletion mutant harboring *cspB*, on the other hand, retained a hemolysis phenotype that was comparable or even marginally better compared to the wild-type strain. A similar phenotypic trend was observed when we assessed the abundance of LLO secreted into culture supernatants of these mutants through comparison of their hemolysis to the wild-type strain (Fig. 2B). The abundance of *hly* encoded mRNA in these strains was also determined. In comparison to the wild-type strain, the *hly* mRNA levels detected in Δ*cspAB* and Δ*cspBD* mutants were significantly ($p < 0.05$; Wilcoxon/Kruskal–Wallis rank-sum test) lower, whereas those in the Δ*cspAD* mutant were similar. Although having similar hemolytic activity levels to the Δ*cspBD*, the Δ*cspAB* mutant cells contained twofold more *hly* mRNA than those of Δ*cspBD*. Overall, these results thus suggest variable contributions of *csp* genes to the modulation of *hly* mRNA levels and LLO activity. CspB seems to be the most significant as it is sufficient to maintain wild-type levels of LLO activity and gene expression. On the other hand, CspA and CspD, if present alone, are unable to sustain wild-type LLO activity and gene expression levels.

Impact of Csps on hly *mRNA stability*

Csps are nucleic acid chaperones that among other functions are presumed to regulate mRNA turnover events in bacteria. To examine whether they could influence LLO production by such functions, the impact of *csp* genes on the stability of *hly* mRNA in *L. monocytogenes* EGDe cells was investigated. The *hly* mRNA half-lives in log-phase wild-type and Δ*cspABD* EGDe cultures in which *de novo* transcript synthesis had been inhibited using rifampicin were compared. As shown in Figure 3, the *hly* mRNA in *L. monocytogenes* cells lacking *csp* genes was more rapidly degraded compared to the wild-type strain. Although in wild-type EGDe cells the log-phase *hly* mRNA half-life is almost 30 min, it was less than 10 min in the absence of *csp* genes in Δ*cspABD*. Low *hly* mRNA levels in *csp* lacking mutants in comparison to the wild-type EGDe strain might thus be due to increased *hly* mRNA turnover caused by the lack of Csps.

Secreted LLO levels in triple and double csp *gene deleted EGDe strains*

To examine if the alteration in *hly* mRNA levels caused by *csp* gene deletions also had an impact on LLO production, the concentrations of LLO secreted in the culture supernatants of

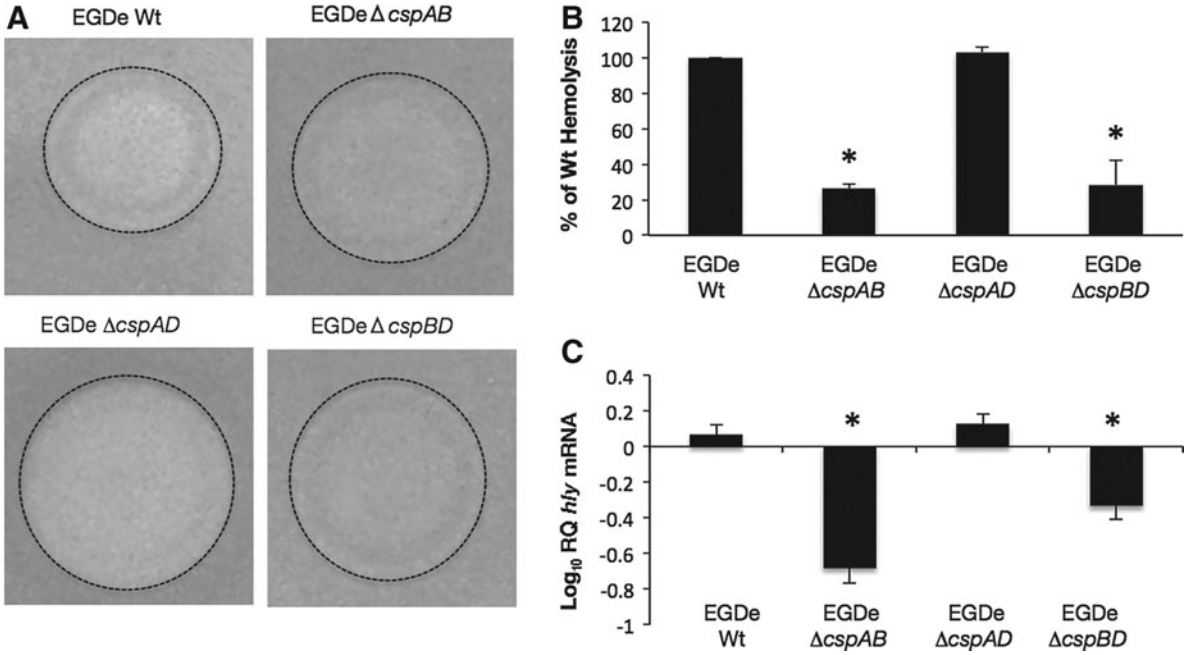

FIG. 2. Hemolysis and gene expression analysis in EGDe wild-type (wt) strain and double *csp* gene deletion (Δ*cspAB*, Δ*cspAD*, *and* Δ*cspBD)* mutants. **(A)** Hemolysis observed on Columbia blood (5%) agar. **(B)** Relative quantification of secreted listeriolysin O hemolytic activity. The hemolysis was determined by adding cell-free supernatants to a 0.025% blood solution prepared in phosphate-buffered saline. The hemolytic activities are expressed relative to that of the parental EGDe wild-type strain (100%). **(C)** Relative quantification of *hly* mRNA. Results presented are the means of three independent experimental runs. Error bars indicate the standard error of the mean. An asterisk denotes statistically significant ($p < 0.05$; Wilcoxon/Kruskal–Wallis rank-sum test) differences in *hly* mRNA levels between the EGDe wild-type and the *csp* deletion mutant strains.

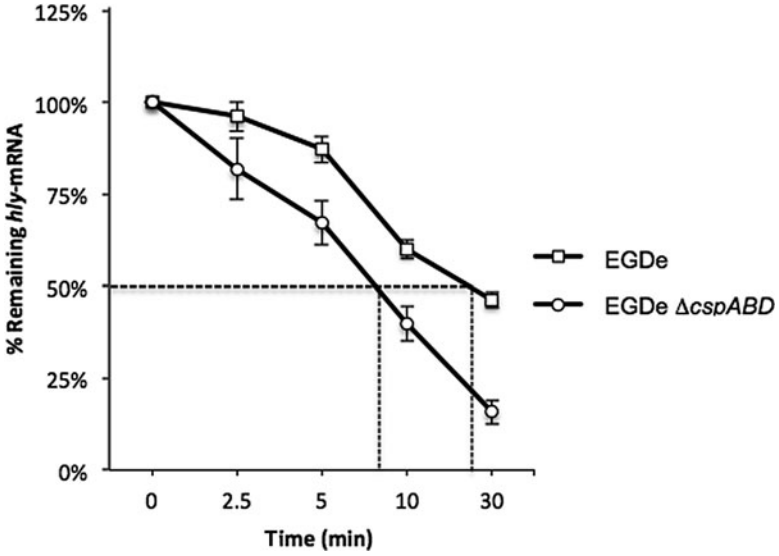

FIG. 3. Effect of cold shock proteins on *hly* mRNA stability as determined by quantitative reverse transcription–polymerase chain reaction. Total RNA was isolated from log phase EGDe wild-type and Δ*cspABD* cells at defined time intervals after addition of rifampicin. Quantities of *hly* mRNA at defined time points following addition of rifampicin were determined relative to *16S* rRNA, and expressed as a percentage of *hly* mRNA quantity that was present at time point zero. Results presented are the means of three independent experimental runs. Error bars indicate the standard error of the mean. Dashed lines indicate *hly* mRNA half-lives.

wild-type, double and triple *csp* gene deletion mutant strains of EGDe were compared. Proteins precipitated from the culture supernatants of these strains were probed by immunoblotting using an anti-LLO antibody. The abundance of LLO proteins detected from the wild-type and the Δ*cspAD* strain harboring *cspB* was comparable (Fig. 4). In contrast, LLO concentrations were lower compared to the wild-type strain in supernatant proteins derived from Δ*cspABD* mutant, which lacks *csp* genes, as well as those derived from the Δ*cspBD* and Δ*cspAB* strains that possess functional *cspA* and *cspD* genes, respectively. These observations are consistent with the hemolysis activity and *hly* mRNA level data, and provide further confirmation of reduced LLO expression in double and triple *csp* deletion mutants.

Discussion

L. monocytogenes mutants devoid of *csp* genes were previously found to exhibit a phenotype that was impaired in virulence. A deletion mutant lacking *cspB* and *cspD* genes or one lacking all three *csp*-encoding genes was severely impaired in epithelial cell and murine macrophage infections (Loepfe *et al*., 2010). These two *csp* mutant strains are also more sensitive to oxidative stress. These observations thus suggest that Csp-dependent gene expression regulation is important in general environmental resistance as well as virulence responses.

It is possible that Csps promote virulence through modulation of oxidative stress gene expression, which is crucial for *L. monocytogenes* survival of vacuolar oxidative stress in macrophages. Alternatively, Csps could regulate expression of yet unknown virulence-associated functions. One such virulence factor might be LLO, which promotes *L. monocytogenes* escape from primary and secondary internalization vacuoles, a potentially useful diagnostic marker (Hamon *et al*., 2012).

Experimental evidence provided in the present study strongly suggested that Csps are involved in mechanisms regulating LLO expression in *L. monocytogenes*. Mutant strains lacking all three *csp* genes or *cspB* only have significantly lower LLO levels compared to the wild-type strain with intact *csp* genes. On the other hand, a mutant possessing only *cspB* gene has LLO at levels that are comparable with the wild-type strain. We thus conclude that Csps are involved in positive regulation of LLO expression and cytolytic activity in *L. monocytogenes* cells. Functional redundancy between Csps in this role is also expected. For example, we found that altered hemolysis phenotypes due to *csp* deletion are only apparent in the context of certain double (Δ*cspAB* and Δ*cspBD*) and triple *csp* deleted mutants. No phenotypic alteration in hemolysis was detected in single *csp* gene deletion mutant variants including that of *cspB*, which appears to be the main *csp* gene involved in promoting LLO

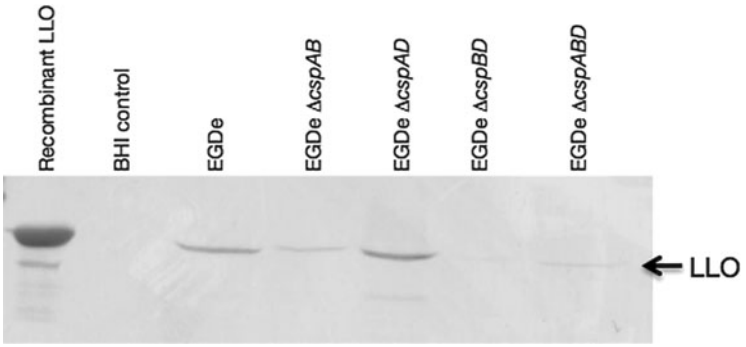

FIG. 4. Immunoblot analysis of listeriolysin O (LLO) proteins secreted by the EGDe wild-type and double (Δ*cspAB*, Δ*cspAD* and Δ*cspBD*) and triple (Δ*cspABD*) *csp* gene deletion mutants. Equal amounts of proteins precipitated from culture supernatants of these strains grown for 16 h in BHI at 37°C were analyzed. As positive and negative controls, 3 μg of the recombinant LLO protein (Lane 1), and a total protein precipitate derived from Brain Heart Infusion broth (BHI) medium (lane 2) were also loaded. A rabbit polyclonal antibody directed against LLO was used for LLO detection. The LLO position is indicated by an arrowhead.

production. However, further studies in the future, including genetic complementation, are needed to clearly define the roles of the different Csps in regulation of LLO gene expression in *L. monocytogenes*.

The mechanisms of Csp-dependent LLO production regulation remain to be understood, although bacterial Csps are known to regulate transcription and translation, as well as mRNA stability (Feng *et al*., 2001; Horn *et al*., 2007; Phadtare and Severinov, 2010). For most of the *csp* deletion mutants, the *hly* mRNA levels detected were lower compared to the wild-type strain. In most *csp* mutants, the reductions in *hly* mRNA levels were consistent with impaired hemolysis and LLO levels. Mutants devoid of all three *csp* genes or *cspB* alone expressed less *hly* mRNA, and showed reduced hemolysis and LLO protein concentrations, compared to wild-type strain. The Δ*cspAD* strain, on the other hand, displayed *hly* mRNA levels that were similar to the wild-type strain. Interestingly, although *hly* mRNA detected in Δ*cspAB* was lower than in Δ*cspBD*, supernatants from this strain contained higher LLO concentrations than those in detected in Δ*cspBD*. We presume this might arise from possible differences in Csp-associated post-transcriptional regulation of LLO production.

It is possible that reduced *hly* mRNA in *csp* mutants arises from altered transcription or mRNA turnover. The CspC and CspE proteins were shown to enhance RpoS and UspA levels, as well as promote increased stability of their encoding mRNAs in *E. coli* (Phadtare and Inouye, 2001). Moreover, *E. coli* CspE was described to bind RNA and impede poly (A)-mediated 3′-5′ mRNA decay due to polynucleotide phosphorylase (Feng *et al*., 2001). The amount of LLO secreted and abundance of its encoding mRNA were significantly decreased due to lack of Csps, in particular CspB. The reduction in LLO encoding *hly* mRNA can in part be explained by its reduced stability detected in absence of Csps. The *hly* mRNA was three times more rapidly degraded in the Δ*cspABD* cells as compared to the wild-type EGDe cells. Thus, one of the mechanisms of Csp-dependent LLO expression promotion depends on their role in modulation of *hly* mRNA stability.

To conclude, the current study provides further evidence that Csps are also involved in modulation of *L. monocytogenes* virulence. It appears that Csp-dependent gene expression regulatory mechanisms that are not yet known also promote LLO production. As shown here, one such mechanism might depend on Csps enhancing *hly* mRNA stability, thereby allowing synthesis of more LLO proteins.

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Disclosure Statement

No competing financial interests exist.

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